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A Novel Polymer-Synthesized Ceramic Composite Based System for Bone Repair: Osteoblast Growth on Scaffolds with Varied Calcium Phosphate Content

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ABSTRACT

Polymer/ceramic composite matrices for bone tissue engineering were constructed by synthesizing a poorly crystalline calcium phosphate within poly(lactide-co-glycolide) microspheres that were subsequently fused together to form 3-dimensional structures. Calcium ion dissolution from the composite matrices in simulated body fluid was examined over a 24 hour period. The initial 4 hour period showed an increase in calcium ion concentration, whereas, a decrease in calcium ion concentration was noted thereafter. This decrease in concentration coincided with the precipitation of calcium phosphate on the surface of the matrices. Osteoblast proliferation studies on composite matrices showed statistically significant increases in cell number throughout the 21 day time period. These data together suggest that the composite matrix acts as both a calcium ion donor for reprecipitation of calcium phosphate that may enhance osteointegration of the implant, and a suitable surface for osteoblast proliferation.

INTRODUCTION

The current gold standard for the treatment of traumatic bone injury is the autograft, but harvesting this graft material requires additional surgery, leading to potential complications and added pain and discomfort for the patient, collectively known as donor-site morbidity. As an alternative, allografts, or tissue taken from cadavers, have been used with suitable levels of success but also with additional concerns and limitations. Allograft tissue is heat treated to minimize the chance of disease transmission from donor to recipient, but this heat treatment results in the destruction of many of the proteins and factors that contribute to healing, ultimately lowering the healing capability of allograft tissue and also altering the intrinsic mechanical properties of the tissue [1]. Further, despite the heat treatment there have been incidents of disease transmission via allograft tissue as recently as 2001 [2]. Tissue engineering has shown great promise as a viable alternative to currently available bone graft solutions due to its use of biocompatible, biodegradable scaffolds as support systems for cellular attachment, proliferation, migration, and maintenance of normal phenotypic expression.

A previous study Khan et al. demonstrated the synthesis of degradable scaffolds from PLGA/calcium phosphate composite microspheres in which an amorphous calcium phosphate is synthesized within the forming microspheres [3]. Analysis of the calcium phosphate formed has shown it to be a poorly crystalline hydroxyapatite according to x-ray diffraction and energy dispersion spectroscopy analysis. Two different ratios of polymer/ceramic were formed; a low polymer/ceramic ratio (27% ceramic) and a high polymer/ceramic ratio (17% ceramic) [3]. The current study examines the release of calcium ions from the poorly crystalline hydroxyapatite in

the composite scaffolds into surrounding media, and the ability of osteoblast-like cells to proliferate on the composite matrices.

EXPERIMENTAL DETAILS

Scaffold Preparation

Scaffolds were prepared as described in detail previously [3]. Briefly, PLAGA/calcium phosphate composite matrices were formed by creating an emulsion of a calcium nitrate tetrahydrate $[\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}]$ solution and ammonium hydrogenphosphate $[(\text{NH}_4)_2\text{HPO}_4]$ solution in a solution of poly(lactide-co-glycolide) (85/15) (Alkermes, Cambridge MA) in methylene chloride. This suspension was added dropwise to 1% polyvinyl alcohol (PVA) (Sigma-Aldrich, St. Louis, MO) (Mw: 30,000-70,000) and allowed to mix at 4°C for 24 hours after which the formed composite microspheres were isolated from the PVA via vacuum filtration and dried at room temperature for 48 hours. Microspheres were then lyophilized for an additional 48 hours. After drying, microspheres ranging from 355-600µm were isolated using stainless steel sieves (Fisher Scientific) and poured into a stainless steel mold to form either cylinders measuring 5mm in diameter and 10mm in length for calcium ion dissolution studies, or discs measuring 14 mm in diameter and 3 mm in thickness for cell proliferation studies. Microspheres were heated at 90°C for 90 minutes to sinter neighboring microspheres together, and subsequently allowed to cool slowly over several hours, resulting in a polymer/ceramic composite scaffold with a porous, interconnected structure.

Calcium Ion Dissolution Studies

Cylindrical matrices were placed in a large excess of simulated body fluid (SBF) (at least 1:100 w/v) and allowed to incubate at 37°C for the following times: 1 hour, 4 hour, 12 hour, and 24 hour. Six samples were incubated for each time point. After each time point, a sample of SBF was removed and analyzed for free calcium ion content. Results were compared to SBF containing no matrices.

Cell Culture Conditions

To minimize the potential for contamination during cell culture, scaffolds were exposed to ultraviolet light for 15 minutes on each side, incubated in two separate 15 minute washes of 70% ethanol and one wash of double distilled water (DD-H₂O) for 5 minutes, and allowed to dry at room temperature overnight. Scaffolds were secured to tissue culture polystyrene using vacuum grease prior to ethanol washes. Primary osteoblast-like cells, MC3T3-E1 (ATCC, Manassas VA) were seeded onto scaffold surfaces at a density of 50,000 cells per well. Cells were allowed to attach for 20 minutes, at which time culture medium was added. Cells were cultured for 21 days in Minimal Essential Medium (MEM) containing 10% fetal bovine serum (FBS), 1% antibiotics (Penicillin/Streptomycin), 3mM β-glycerophosphate (as a phosphate donor), and 10µg/ml ascorbic acid. Cells were analyzed after 3, 7, 14, and 21 days of growth. Media was changed every other day and cells were kept at 37°C in a humidified environment of 5% CO₂. Each group (as mentioned under experimental set-up) was repeated in triplicate.

Experimental Set-up

Cell culture experiments consisted of 4 different experimental groups:

- 1- tissue culture polystyrene (TCPS)
- 2- pure PLAGA microspheres

- 3- composite microsphere matrices with a low polymer/ceramic ratio
- 4- composite microsphere matrices with a high polymer/ceramic ratio

Proliferation Assay

Cellular proliferation was determined using an MTS assay (Promega, Inc. San Luis Obispo CA) that was allowed to react with cell cultures while cells were still attached to the matrices, for 2 hours at 37°C and 5%CO₂, at which time the reactant was transferred to a separate vessel where optical density of the reactant was measured at 492 nm. Results were compared to a standard curve and converted to number of cells adhered to the matrix.

Statistics

Results of proliferation were analyzed using a one-way analysis of variance (ANOVA) and the Tukey post hoc test for differences within groups between time points. P values were set at 0.05.

DISCUSSION

Bone is a dynamic tissue that is constantly undergoing a process of resorption, synthesis, and remodeling and serves as both a source and sink for physiological calcium. The synergistic activity between osteoclasts and osteoblasts provide the tools for this ongoing process, in which osteoclasts resorb the bone, liberating calcium and phosphate ions, and osteoblasts secrete osteoid that is mineralized in part by using the locally available calcium and phosphate ions.

Therefore, it can be surmised that a local supply of available calcium and phosphate ions may enhance the tendency for a bone graft substitute to be incorporated into the surrounding bone. This notion can prove valuable when designing bone graft substitutes, in which the incorporation of a calcium phosphate may prove beneficial.

The use of calcium phosphate in a bone graft substitute is well reasoned as it is a form of calcium phosphate, specifically poorly crystalline calcium deficient hydroxyapatite that forms the inorganic component of bone. Further, calcium phosphates have shown excellent biocompatibility, a high degree of bone ingrowth, and enhanced bone formation when used as bone defect fillers [4-6]. Many currently available implant materials use a highly crystalline, poorly resorbable hydroxyapatite that liberates ions very slowly over long periods of time. Using a less crystalline calcium phosphate would allow for greater liberation of calcium ions which may lead to enhanced healing. Studies examining this relationship between crystallinity and healing have shown an increase in mineral formation and bone integration on calcium phosphates with decreasing crystallinity [7, 8].

Calcium Ion Dissolution Studies

Composite matrices of either high or low polymer/ceramic ratio were analyzed for calcium ion dissolution from the poorly crystalline hydroxyapatite within the matrix. Results showed an initial increase of Ca²⁺ after 1 and 4 hours of incubation as compared to SBF alone, indicating that Ca²⁺ ions were being released into the SBF (Table 1). However, this trend changed to decrease in Ca²⁺ ion concentration after 12 hours of incubation, and continued for the 24 hour time point. It was thought that this shift in ion concentration may be due to the fact that as the Ca²⁺ ion concentration increased, calcium phosphate began to precipitate on the surface of the composite matrices. This finding was supported by scanning electron micrographs showing abundant calcium phosphate precipitation after only 3 days of incubation (images not shown). There was little or no difference in ion dissolution between the low and high ratio composite

matrices, suggesting that the amount of ion dissolution was not dependent on the initial availability but possibly dependent on an overall concentration gradient. It could be argued that the calcium phosphate that precipitated was hydroxyapatite as the SBF became more acidic over time. With the formation of hydroxyapatite, hydroxyl ions were incorporated as well as calcium and phosphate ions, which would suggest that the pH would drop, as was seen here (data not shown).

Table 1. Calcium ion release from composite matrices of either low or high polymer/ceramic ratio into simulated body fluid over 24 hours. Values indicate percent of control SBF solution. Initial increases in free Ca^{2+} are quickly replaced by decreases on both matrix types after 12 and 24 hours, suggesting the rapid precipitation of calcium phosphate on the surface of the matrices.

Matrix Type	PERCENT Ca^{2+} OF SIMULATED BODY FLUID			
	1 hour release	4 hour release	12 hour release	24 hour release
Low Ratio	123.71 \pm 11.3	137.78 \pm 10.59	82.14 \pm 3.66	66.12 \pm 2.38
High Ratio	122.61 \pm 8.98	136.83 \pm 6.46	86.77 \pm 6.01	81.03 \pm 6.07

Cell Proliferation on Composite Microsphere Matrices

MC3T3-E1 cells seeded onto matrices composed of either pure PLAGA microspheres or composite microspheres showed statistically significant increases in proliferation through the course of the 21 day analysis (**Figure 1**). Cells seeded on TCPS also showed statistically significant increases at each subsequent time point except between 7 and 14 days. Cell proliferation was lower on the high polymer/ceramic ratio matrices than on the low polymer/ceramic matrices at each time point. It is also noted that cell number on both the low and high polymer/ceramic composite microsphere matrices was lower than that of either TCPS or PLAGA matrices at each time point but cells continued to proliferate over the 21 day analysis despite the apparent initial low cell number at the earlier time points.

Also noted was a slight drop in pH of the media in wells containing composite matrices. This drop may be due to the release of calcium ions and subsequent reprecipitation of hydroxyapatite onto the matrix surface. The reprecipitation of hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6\text{OH}_2$] would occupy hydroxyl ions, leading to a decrease in pH. This decrease in pH may create a slightly cytotoxic environment for the cells, leading to lower cell numbers at early time points, with a gradual recovery due to proliferation of existing cells, as is seen in the later time points. Parallel studies are examining the effects of the composite matrices on pH balance. The reduction in cell number seen on composite matrices may also be due to limited spreading of cells on the calcium phosphate surface. Other studies have shown limited colony occupancy of osteoblasts cultured on nanophase ceramics including hydroxyapatite [9]. According to x-ray diffraction patterns of the calcium phosphate formed within the composite matrices, there is good evidence that the calcium phosphate formed is nanophase hydroxyapatite [6]. Therefore if a certain percentage of the microsphere surface was nanophase hydroxyapatite, the osteoblasts may have been restricted in migration, and thus have been limited in proliferation.

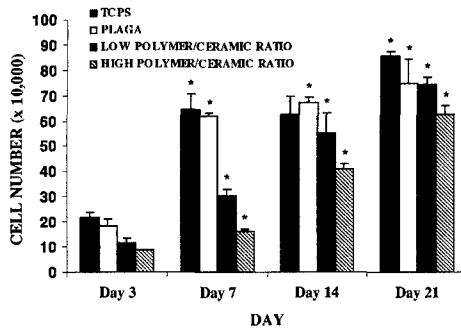


Figure 1. Proliferation of osteoblasts seeded onto TCP, pure polymeric matrices, or composite matrices. Significant increases in proliferation were seen on all matrices over the 21 day study. ‘*’ Indicates significance when compared to the respective culture surface for the previous time point ($p < 0.05$).

CONCLUSION

Composite matrices were formed by synthesizing a poorly crystalline calcium phosphate within forming poly(lactide-co-glycolide) microspheres and sintering the formed microspheres together to form 3-dimensional structures. Calcium ion dissolution from the poorly crystalline calcium phosphate contained within the microspheres was quantified and found to increase up to 4 hours of incubation in simulated body fluid but then decreased during the subsequent 20 hours of incubation, suggesting that the poorly crystalline calcium phosphate was inducing reprecipitation of calcium phosphate on the surface of the microspheres. Osteoblast-like cell proliferation studies show abundant cell proliferation on matrix surfaces throughout the 21 day study. These data taken together suggest that the composite matrices may serve as a viable material for bone tissue engineering that would both support osteoblast attachment and growth as well as encourage osteointegration through the reprecipitation of calcium phosphate.

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